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14. ABSTRACT

Angiogenesis and metastasis are two processes that are central to the progression of cancer. As such, they have become important targets for the development of anti-cancer agents. Invasive and metastatic cancers of the breast are distinguished by their propensity of newly formed blood vessels (neoangiogenesis). Neoangiogenesis is a significant independent prognostic indicator in early stage breast cancer (1). Delineating the molecular mechanism(s) of neoangiogenesis may provide new insights into the biology of breast cancer progression and metastasis and may provide novel prognostic and therapeutic tools. Recently, the plasminogen (PLG)/plasmin (PL) system was demonstrated to play an important role in breast cancer progression and metastasis. Experimental studies in animal models combined with extensive clinicopathological data provide a compelling case indicating that proteins of PLG/ PL pathways play a key role in breast cancer progression and metastasis(2). In this context, enzymes of the PLG/PL pathway have been reported to have prognostic value in breast cancer and are associated with poor prognosis both for overall and disease free survival(2). In fact these molecules have been associated with a high rate of relapse for patients with breast cancer Preliminary studies in animal model demonstrated that PLG gene deficient mice (PLG-/-) display inhibition of tumor invasion, lymph node metastasis and angiogenesis supporting the idea that PLG is required for angiogenesis, tumor growth and metastasis(3). Despite established role in tumor angiogenesis, growth and metastasis it is still unclear how

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INTRODUCTION

Sprouting of new blood vessels and metastasis are two processes that are central to the progression of cancer. As such, they have become important targets for the development of anti-cancer agents. The plasminogen/plasmin system, a serine protease pathway, is known to trigger sprouting of new blood vessels therefore plays a critical role in breast cancer (1). Analysis of the enzymes of the plasminogen/plasmin system clearly suggests that high urokinase-type plasminogen activator (uPA) in human breast tumors predicts poor survival (2, 3) and is significantly associated with a high rate of relapse for patients with breast cancer (2). Despite the established role of the plasminogen/plasmin system in breast cancer, the receptor(s) which regulates conversion of zymogen plasminogen to active enzyme plasmin in breast cancer are poorly understood. In the physiological system two different mechanisms of plasmin generation have been established. The first mechanism is involved in binding of uPA to its receptor uPAR while the second mechanism converts plasminogen to plasmin by binding of tissue type plasminogen activator (tPA) to its receptor annexin II. This annexin II dependent plasmin exerts fibrinolytic activity that maintains continuous blood flow in vessels by dissolving intravascular fibrin clots.

Annexin II is reported to be an endothelial cell receptor which provides binding sites for plasminogen and tPA for efficient plasmin generation (4, 5). A number of reports have shown that cell surface annexin II regulates plasmin generation (5, 6) which in turn facilitates extracellular matrix (ECM) degradation, cell invasion (6-8), and migration (9) and thus plays an important role in neoangiogenesis (10). There are speculations that annexin II derived neoangiogenesis may be involved in metastasis, and tumor progression. This might explain why we and other investigators have consistently found over expression of annexin II in various cancers including breast cancer (11-19). Overexpression of annexin II in cancer seems to correlate with advanced stage with poor prognosis and metastasis (11, 17, 18, 20).

Breast cancer growth and metastasis requires extensive neoangiogenesis. The fact is that neoangiogenesis is an independent and highly significant prognostic indicator for overall and relapse-free survival in patients with early-stage breast carcinoma (21). Angiostatin, a potent inhibitor of angiogenesis, was reported to inhibit human breast cancer growth and metastasis in xenograft model (22, 23) suggesting that anti-angiogenic therapy could be a potential therapeutic approach for breast cancer. Previously we have reported that angiostatin binds to endothelial cell surface annexin II (24). These findings, together with observations that angiostatin inhibits neoangiogenesis dependent growth of breast cancer may argue that angiostatin inhibits neoangiogenesis dependent breast cancer growth and metastasis by interfering with annexin II/tPA dependent plasmin generation in tumor microenvironment.

tPA is secreted by endothelial cells (EC) and is a ligand for cell surface annexin II. Emerging reports and clinical observations link tPA to neoplastic transformation and the invasive phenotype of highly aggressive tumors such as glioblastoma (25), melanoma (26) pancreatic cancer (27) and breast cancer (28, 29). The functional role of tPA and its receptor annexin II in breast tumor biology is not well-understood. Recent studies have provided evidence that showed association of tPA with the invasion of highly aggressive MDA-MB-435s breast cancer cells (30). Since tPA plays a major role in plasmin generation, consistently plasmin has been linked to invasive and metastatic breast cancer (1). Indeed, recent studies have clearly demonstrated over expression of annexin II in many cancer types and may argue that annexin II functions as a

regulator of neoangiogenesis *in vivo*. However the precise mechanism by which annexin II is involved in cancer progression and metastasis is still unclear.

In this report we investigated if tPA interaction on breast cell surface annexin II is a molecular switch to regulate localized plasmin generation *in vitro* and neoangiogenesis *in vivo*. Taken together, this research may provide a clue for the annexin II-dependent mechanism of neoangiogenesis and its dependent breast cancer progression and metastasis. Annexin II may be a potential target for the development of effective therapeutic strategies to inhibit angiogenesis and breast cancer.

BODY

We have successfully completed task 1 of this idea award as proposed. Part of the results of task1 has been reported in 2008 and 2009 annual report. In 2009 our lab has moved from the Drexel University to the VA Medical center, Washington DC. This move has caused disruption of our research activities. However despite of this disruption, we were able to complete some experiments as we outlined in our task 2 of the SOW. Detailed staining and data analysis is still going on which will be reported after completion of the grant in November, 2011. One experiment as outlined in task 1 of SOW that is evaluation of therapeutic efficacy of anti-ANX II antibody in breast cancer metastasis is underway and will be completed in January 2011. The results obtained in these studies has been published in *Experimental and Molecular Pathology 88 (2010) 278–286 and* one paper was presented in annual meeting of *American Association for Cancer Research Proc. Am. Assoc. Can. Res. (2010) 15, 313*(Full manuscript is being submitted for publication). We published one review article in *Current Pharmaceutical Design*, 2009, *15,* 1949-1955 (see attached reprints). The results are summarized below.

KEY ACHIEVEMENTS:

Task2: To determine whether the pattern of Annexin II expression in breast cancer correlates with neoangiogensis and worsening stage of breast cancer and predictive of poor outcome.

One experiment planned in task1d entitled effect of anti-annexin II antibody on mouse model of breast cancer metastasis is in underway and likely to be completed in January 2011. Results will be reported in final report

To investigate the role of annexin II in human breast cancer progression we analyzed human breast tissue samples by immunohistochemical staining. Immunohistochemical studies revealed expression of ANX II mainly on the surface of invasive cancerous cells (Fig. 1B). In contrast, epithelial cells in normal ducts were completely negative (Fig. 1G). Consistent with annexin II expression, immunostaining of

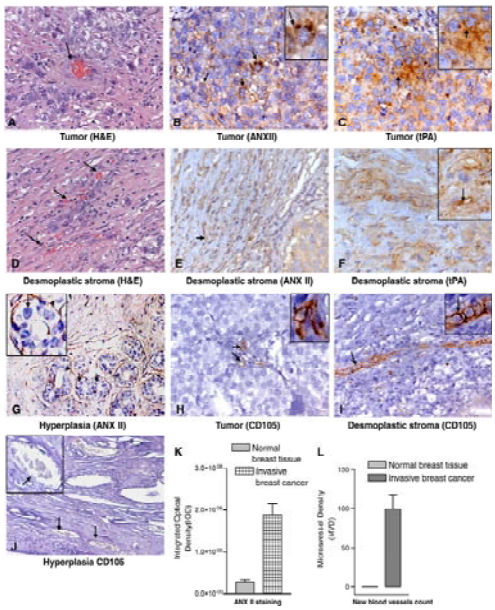

Fig. 1. Immunostaining for annexin II, tPA and anti-CD 105 in normal and human breast cancer tissues. Annexin II is undetectable in normal acinar and ductal epithelial cells (G, inset arrows). Anti-annexin II antibodies show a very strong reactivity on the surface of ductal epithelial cells (B, inset), tumor stroma (E) and vascular endothelial cells (B, arrows) in invasive breast cancer. Staining in ductal epithelial cells was found mainly on the cell surface with occasional cytoplasmic staining. Anti-tPA antibodies show intense staining in vascular endothelial cells(F inset), ductal epithelial cells (C, inset) and in tumor stroma. Anti-CD105 antibodies show a selective strong reactivity with newly formed blood vessels (H) but not in existing blood vessels of normal tissue (J). Panel J arrows indicate existing blood vessels in normal breast tissue are not stained with anti-CD105 antibody suggesting specificity of CD105 to identify proliferating endothelial cells (Magnification 20X). Staining intensity of annexin II was analyzed in 20 different fields by ImagePro Software Panel K.CD 105 positive endothelial cells were counted and plotted by GraphPad Prizm software Panel L.

thin serial sections also revealed high levels of tPA in tumor stroma (Fig. 1C). Careful investigation of immunostained sections revealed collagenous stroma composed of fibroblasts, macrophages, lymphocytes and ECM indicating an inflammatory response and annexin II in desmoplastic stroma (Fig. 1E) suggesting chronic inflammation in the tumor microenvironment and may be indicative of neoangiogenic activity. Staining pattern of CD105 revealed numerous sprouting neovessels intumor as well in desmoplastic stroma confirming neoangiogenic activity (Fig. 1H and I). Quantitative analysis of neoangiogenic activity as measured by micro vascular density (MVD) appears to correlate with annexin II expression patterns (Fig. 1K and L).

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1. Annexin II expression is appeared to be on the surface of aggressive human breast cancer patients. Very low or no expression was found on normal ducts.
2. Annexin II expression seems to correlate with neoangiogenic activity in human breast cancer patients.

REPORTABLE OUTCOMES: The results of this study have been published in following Journals/meetings.

1. *Experimental and Molecular Pathology 88 (2010) 278–286.One paper was* presented in annual meeting of *American Association for Cancer Research*

2. *Proc. Am. Assoc. Can. Res. (2010) 15, 313* (Full manuscript is being submitted for publication). One review article was published in
3. *Current Pharmaceutical Design,* 2009, *15,* 1949-1955

CONCLUSIONS:

Our Immunohistochemical staining results indicates selective expression of annexin II in invasive breast cancer specimens. Annexin II expression appears to correlate with neoangiogenic activity in invasive breast cancer patients.

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Breast cancer cell surface annexin II induces cell migration and neoangiogenesis via tPA dependent plasmin generation

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ABSTRACT

Annexin II, an abundant phospholipids binding cell surface protein, binds tPA and functions as a regulator of fibrinolysis. Annexin II also mediates angiogenesis and enhances tumor growth and metastasis. However, the mechanism supporting this role is not known. Using human breast cancer model we show that invasive human breast cancer cells (MDA-MB231) synthesize annexin II and tissue plasminogen activator (tPA). *In vitro* both annexin II and tPA interacts which in turn convert zymogen plasminogen to reactive enzyme plasmin. Cell surface produced plasmin inhibited the migration of MDA-MB231 cells. Silencing of annexin II gene in MDA-MB231 cells abolished tPA binding therefore inhibited tPA dependent plasmin generation. These annexin II suppressed MDA-MB231 cells showed reduced motility. Immunohistochemical analysis of prediagnosed clinical specimens showed abundant secretion of tPA and expression of annexin II on the surface of invasive human breast cancer cells which correlates with neovascularization of the tumor. Taken together, these data indicate that annexin II may regulate localized plasmin generation in breast cancer. This may be an early event switching breast cancer from the prevascular phase to the vascular phase and thus contributing to aggressive cancer with the possibility of metastasis. The data provide a mechanism explaining the role of annexin II in breast cancer progression and suggest that annexin II may be an attractive target for therapeutic strategies aimed to inhibit angiogenesis and breast cancer.

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Introduction

Sprouting of new blood vessels and metastasis are two processes that are central to the progression of cancer. As such, they have become important targets for the development of anti-cancer agents. The plasminogen/plasmin system, a serine protease pathway, is known to trigger sprouting of new blood vessels therefore plays a critical role in breast cancer (Stephens et al., 1998). Analysis of the enzymes of the plasminogen/plasmin system clearly suggests that high urokinase-type plasminogen activator (uPA) in human breast tumors predicts poor survival (Janicke et al., 1989; Look et al., 2002) and is significantly associated with a high rate of relapse for patients with breast cancer (Janicke et al., 1989). Despite the established role of the plasminogen/plasmin system in breast cancer, the receptor(s) which regulates conversion of zymogen plasminogen to active enzyme plasmin in breast cancer are poorly understood. In the physiological system two different mechanisms of plasmin generation have been established. The first mechanism is involved in binding of uPA to its receptor uPAR while the second mechanism converts plasminogen to plasmin by binding of tissue type plasminogen activator (tPA) to its receptor annexin II. This annexin II dependent plasmin exerts fibrinolytic activity that maintains continuous blood flow in vessels by dissolving intravascular fibrin clots.

Annexin II is reported to be an endothelial cell receptor which provides binding sites for plasminogen and tPA for efficient plasmin generation (Cesarman et al., 1994; Hajjar et al., 1994). A number of reports have shown that cell surface annexin II regulates plasmin generation (Diaz et al., 2004; Hajjar et al., 1994) which in turn facilitates extracellular matrix (ECM) degradation, cell invasion (Brownstein et al., 2004; Diaz et al., 2004; Mignatti and Rifkin, 1993), and migration (Tarui et al., 2002) and thus plays an important role in neoangiogenesis (Ling et al., 2004). There are speculations that annexin II derived neoangiogenesis may be involved in metastasis, and tumor progression. This might explain why we and other investigators have consistently found over expression of annexin II in various cancers including breast cancer (Chuthapisith et al., 2009; Guedj et al., 2009; Ohno et al., 2009; Pei et al., 2007; Sharma et al., 2006a; Shiozawa et al., 2008; Syed et al., 2007; Zhong et al., 2009; Zimmermann et al., 2004). Overexpression of annexin II in cancer seems to correlate with advanced stage with poor prognosis and

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metastasis (Ohno et al., 2009; Sharma et al., 2006a; Shiozawa et al., 2008; Wu et al., 2002).

Breast cancer growth and metastasis requires extensive neoangiogenesis. The fact is that neoangiogenesis is an independent and highly significant prognostic indicator for overall and relapse-free survival in patients with early-stage breast carcinoma (Weidner et al., 1992). Angiostatin, a potent inhibitor of angiogenesis, was reported to inhibit human breast cancer growth and metastasis in xenograft model (O'Reilly et al., 1996; Peyruchaud et al., 2003) suggesting that anti-angiogenic therapy could be a potential therapeutic approach for breast cancer. Previously we have reported that angiostatin binds to endothelial cell surface annexin II (Tuszynski et al., 2002). These findings, together with observations that angiostatin inhibits neoangiogenesis dependent growth of breast cancer may argue that angiostatin inhibits neoangiogenesis dependent breast cancer growth and metastasis by interfering with annexin II/tPA dependent plasmin generation in tumor microenvironment.

tPA is secreted by endothelial cells (EC) and is a ligand for cell surface annexin II. Emerging reports and clinical observations link tPA to neoplastic transformation and the invasive phenotype of highly aggressive tumors such as glioblastoma (Goh et al., 2005), melanoma (Stack et al., 1999) pancreatic cancer (Diaz et al., 2002) and breast cancer (Grondahl-Hansen et al., 1990; Rella et al., 1993). The functional role of tPA and its receptor annexin II in breast tumor biology is not well-understood. Recent studies have provided evidence that showed association of tPA with the invasion of highly aggressive MDA-MB-435s breast cancer cells (Chernicky et al., 2005). Since tPA plays a major role in plasmin generation, consistently plasmin has been linked to invasive and metastatic breast cancer (Stephens et al., 1998). Indeed, recent studies have clearly demonstrated over expression of annexin II in many cancer types and may argue that annexin II functions as a regulator of neoangiogenesis *in vivo*. However the precise mechanism by which annexin II is involved in cancer progression and metastasis is still unclear.

In this report we investigated if tPA interaction on breast cell surface annexin II is a molecular switch to regulate localized plasmin generation *in vitro* and neoangiogenesis *in vivo*. Taken together, this research may provide a clue for the annexin II-dependent mechanism of neoangiogenesis and its dependent breast cancer progression and metastasis. Annexin II may be a potential target for the development of effective therapeutic strategies to inhibit angiogenesis and breast cancer.

Materials and methods

Human Lys-plasminogen, plasmin and recombinant tPA was purchased from Calbiochem, (La Jolla, CA). Lysine–Sepharose and ε-aminocaproic acid (ε-ACA) were procured from Sigma (St. Louis, MO). Chromatography and electrophoretic reagents were procured from BioRad, (Richmond, CA). Anti-plasminogen monoclonal antibody was purchased from Enzyme Research Inc. (Chicago, IL). Anti-tPA and Anti-CD105 monoclonal antibodies were purchased from American Diagnostica (Stamford, CT) and Neomarkers (Fremont, CA) respectively. Antibodies to annexin II were generated in our lab as reported earlier (Sharma et al., 2006b). Chromozyme PL was purchased from Roche Molecular Biochemicals (IN). Immunohistochemistry reagents were procured from DAKO Corporation (CA). All other chemicals used in this study were of analytical grade.

Cell lines maintenance

Invasive and metastatic human breast cancer cell line MDA-MB231 which is known to cause tumors in athymic mice and the ER positive noninvasive breast cancer cell line MCF-7 were obtained from ATCC (Rockville, MD). These cell lines were maintained in RPMI 1640 media containing either 10% fetal calf serum (FCS) or serum free medium (0.1% BSA supplemented with L-glutamine and antibiotics) (Sharma et al., 2006a). Cell cultures were maintained in plastic flasks and incubated at 37 °C in a humidified chamber containing 5% CO_2.

Immunohistochemistry

Previously diagnosed paraffin-embedded human breast cancer samples were obtained from the tumor bank of the Department of Pathology, Drexel University College of Medicine. Serial 4 μm sections were prepared on albumin-coated slides. Sections were deparaffinized and incubated in 3% H_2O_2 for 10 min to block endogenous peroxidase activity. Nonspecific protein binding was blocked with 3% bovine serum albumin (BSA)/PBS for 1 h. Sections were incubated with monoclonal antibodies overnight at room temperature. Staining was visualized using a DAKO kit with diaminobenzidine (DAB) as a chromogen followed by nuclear counterstaining with hematoxylin according to our published protocol (Sharma et al., 2006a). For each immunohistochemical staining, we performed additional staining without primary antibody in parallel and considered it as a control. The immunoreactive pattern was carefully recorded with the help of our pathologist and photographed.

Immunoprecipitation and Western blot analysis

Cells grown in 12 well-plates, rinsed in ice-cold phosphate-buffered saline (PBS), pH 7.2, and lysed in 67 mmol/L Tris, pH 6.8, and 2% sodium dodecyl sulfate on ice for total extracts or in buffer A [50 mmol/L Tris, 150 mmol/L NaCl, pH 7.4, 0.1% Triton X-100 plus protease and phosphatase inhibitors (200 mmol/L Pefabloc, 1 mmol/L aprotinin, 20 mmol/L leupeptin, 0.1 mmol/L sodium vanadate, 1 mmol/L sodium fluoride, and 10 mmol/L sodium pyrophosphate)] for immunoprecipitation. Protein concentration was measured using Bradford assay (Bio-Rad Laboratories, Hercules, CA). Immunoprecipitation were performed using 0.4 to 1 mg of cellular proteins incubated with anti-annexin II monoclonal antibody (0.5 μg/ml) overnight at 4 °C. The immune complex was precipitated with protein A Sepharose beads. The immunocatcher system was used to purify immunoglobulin complex by catching the resins containing immobilized Protein A. Resins were washed three times and directly incubated with SDS sample dilution buffer and heated at 90 °C for 4 min. Immune complex was resolved on 12% SDS-PAGE, transferred to nitrocellulose membrane, incubated with ant-tPA antibodies and analyzed by Western blot analysis. After incubation with the appropriate primary antibody, species-specific secondary antibodies conjugated to horseradish peroxidase were used and the membrane was developed by ECL as we have reported previously (Sharma et al., 2006a,b; Tuszynski et al., 2002).

tPA binding and plasmin generation

Binding of tPA with breast cancer cell (MDA-MB 231) surface was studied by analyzing the capacity of cell bound tPA to activate plasminogen to plasmin. Human breast cancer cells were cultured, detached and counted. About 5000–7000 cells were incubated with 300 units of recombinant human tPA ligand for 30 min on ice in PBS to allow tPA to bind cell surface annexin II. Cells were washed three times with chilled PBS to remove unbound tPA. Breast cancer cells with bound tPA were incubated with plasminogen and specific chromogenic substrate chromozyme PL in a total volume of 100 μl to determine plasminogen activation activity. tPA dependent plasmin generation was recorded at 405 nm for 40 min in 96-well-plate reader according to our published protocol (Sharma et al., 2006a,b). Plasmin generated in the reaction mixture hydrolyzes the chromozyme PL forming a yellow color of p-nitroaniline that absorbs light at 405 nm. The color absorbance was measured by an automated 96-well plate reader (Bio-TeK Inc., VT). The colorimetric change is a direct measurement of plasmin generation. Plasminogen incubated with chromozyme PL was considered as negative control indicative of auto degradation of plasminogen to

plasmin during the reaction process. In a parallel experiment an equal amount of tPA unbound to cells were used as control. Absorbance of control was subtracted from experimental readings. Data were analyzed by non-linear regression algorithms using GraphPad Prizm software.

Annexin II gene silencing

Antisense phosphorothionate oligonucleotides (ODNs) and sense ODNs (control) directed to 5′–3′coding sequence of the human annexin II mRNA were designed and manufactured by Biognostic, Germany (www.biognostik.com). Three potential sites were identified in annexin II gene and antisense oligonucleotides were synthesized against these regions (5′CAA CAT GTC CAC TTC ACT - 3′ location: 923-940 bases of total sequence) (5′ - CAT TTT CCA GGT CTC CTT - 3′ location: 882-899 bases of total sequence) (5′ - TCA TCC ACA CCT TTG GTC - 3′ location: 277–294 bases of total sequence). The antisense oligonucleotides were synthesized as 18-mer targeted annexin II gene sequences. The corresponding controls sequence was also synthesized as 18 mer (5′GGA TTT ACC TAT TGC TGG 3′) (5′ ACT ACG ACC TAC GTG AC 3′) (5′GGA TTT ACC TAT TGC TGG 3′). Control antisenses were used in all parallel experiments. Hereafter, the antisense and sense annexin II oligonucleotides will be referred to as RNAi ANX II and Control ANX II respectively. MDA-MB231 cells were grown in 96-well plates in triplicates and transfected with RNAi ANX II and control ANX II by adding into the culture medium.

Antisense ODNs are actively taken up by cells, partially through fluid-phase endocytosis and possibly also through the putative receptor protein p80 that facilitates the cellular uptake of negatively charged molecules like ODNs or heparin. Fluorescein (FITC)-labeled phosphorothioate ODNs were used to monitor cellular uptake and distribution (data not shown). Labeled antisense ODNs met the same standards of purity and stability as antisense products. Whereas the cellular uptake of antisense ODNs may be enhanced through various cationic lipids, most of the cationic lipids are cytotoxic, and the treatment must be limited to 6 to 8 h. In contrast, the half-life of antisense ODNs in serum containing culture media is >48 h. Therefore, adding the antisense ODNs to the culture medium for the full duration of the experiment is more effective for experiments with a longer time frame. Details of the transfection process are provided below.

Transfection

To optimize transfection efficiencies MDA-MB231 cells were incubated with 3 different concentrations of RNAi ANX II and control ANX II (0.5, 2.0 and 4.0 μmol/L) for 12, 24, 48, and 72 h to determine the rate of transfection. Our optimizing experiments showed that the uptake of RNAi ANX II was maximal with 0.5 μmol/L and after incubation for 48 h. At this time point, annexin II protein was suppressed >90% (see results). Therefore we used these optimum conditions to suppress annexin II gene to determine tPA bindings, it's dependent plasminogen activation and role in cell migration.

Scratch wound healing assay for cell migration

Cell migration was assayed as described (Sharma et al., 2004). Briefly, about 8000 cells were seeded and grown to confluency in 96 well

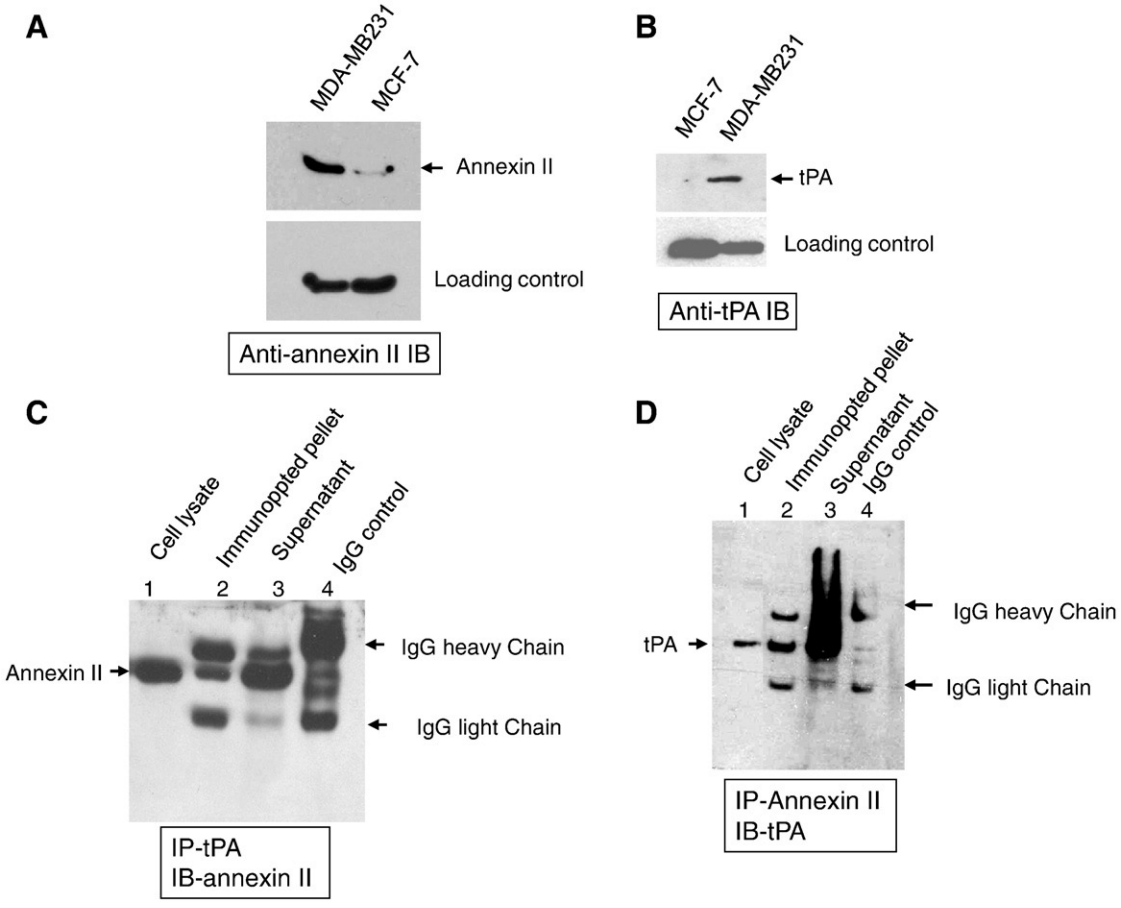

Fig. 1. Expressions and interactions of annexin II and tPA in human breast cancer cells. MDA-MB231cell lysate (20 μg) was separated by SDS-PAGE and protein expression was analyzed by immunoblot analysis. Fig. 2 displays selective expression of annexin II and tPA in invasive breast cancer cell line MDA-MB231 (Fig. 2A and B). Cell lysate was immunoprecipitated (IP) with anti-tPA antibodies and the immune complex was electrophoresed and immunoblotted with anti-annexin II (Panel 2C, Lane 1 positive control, lane 2 IP pellet, lane 3 supernatant and lane 4 is IgG negative control). Panel D: cell lysate was immunoprecipitated (IP) with anti-annexin II antibodies and the immune complex was electrophoresed and immunoblotted with anti-tPA (Panel C, Lane 1 positive control, lane 2 IP pellet, lane 3 supernatant and lane 4 is IgG negative control). Co-immunoprecipitated pellet in lane 2 of both gels suggest interaction of tPA and annexin II.

plates. A wound was created in monolayer of cells by scratching with a sterile pipette tip. The remaining adherent cells were washed twice with PBS. Media was replaced with 2% serum-supplemented medium. Cell motility was measured by counting the number of cells repopulated in the cleared area. Ten different fields (20X) were viewed to count migrating cells. Data were plotted using GraphPad Prizm software.

Boyden chamber migration assay

The measurement of cell migration was performed as described by (Terranova et al., 1986). Chemotaxis cell migration assay was performed using a 96 well plate fitted with 8 μm pore Boyden migration chambers (CHEMICON, Inc. cat # ECM510). Briefly, cells starved in serum free medium were harvested and plated at a density of 2.5×10^4 cells in 100 μl without chemo-attractant in a Boyden chamber (upper well) in a 96 well plate. This assay is based on the Boyden chamber principle. The bottom well was filled (100 μl) with medium containing 10% fetal bovine serum (FBS). Test reagents (plasminogen, plasmin, angiostatin, ε-aminocaproic acid, or antibodies) were added to the upper chamber and incubated for 24 h at 37 °C in a humidified chamber (5%CO). After 24 h, the plate was flipped to remove the cells and medium. Migrated cells adhered on the other side of membrane were detached using detachment buffer and lysed in medium containing a fluorescent dye according to manufacturer instructions. The plate was read in a fluorescence plate reader (Bio-Tek, Inc.,VT) using a 480/520 nm filter set. Serum-free medium alone was used as a negative control. Purified human plasmin was used as positive control. Separate controls were used against each reagent added. For example, mouse IgG was used as control for anti-annexin II antibody, reduced angiostatin was used as control for angiostatin and buffer in which ε-aminocaproic acid was dissolved was used as a control for ε-aminocaproic acid. Statistical analysis and graphic presentation of the data were performed with GraphPad Prizm software.

Results

Cell surface annexin II interacts with recombinant human tPA in human breast cancer MDA-MB231cells

Previous clinical studies including from our laboratory have shown over expression of annexin II in cancer patients. Over expression has

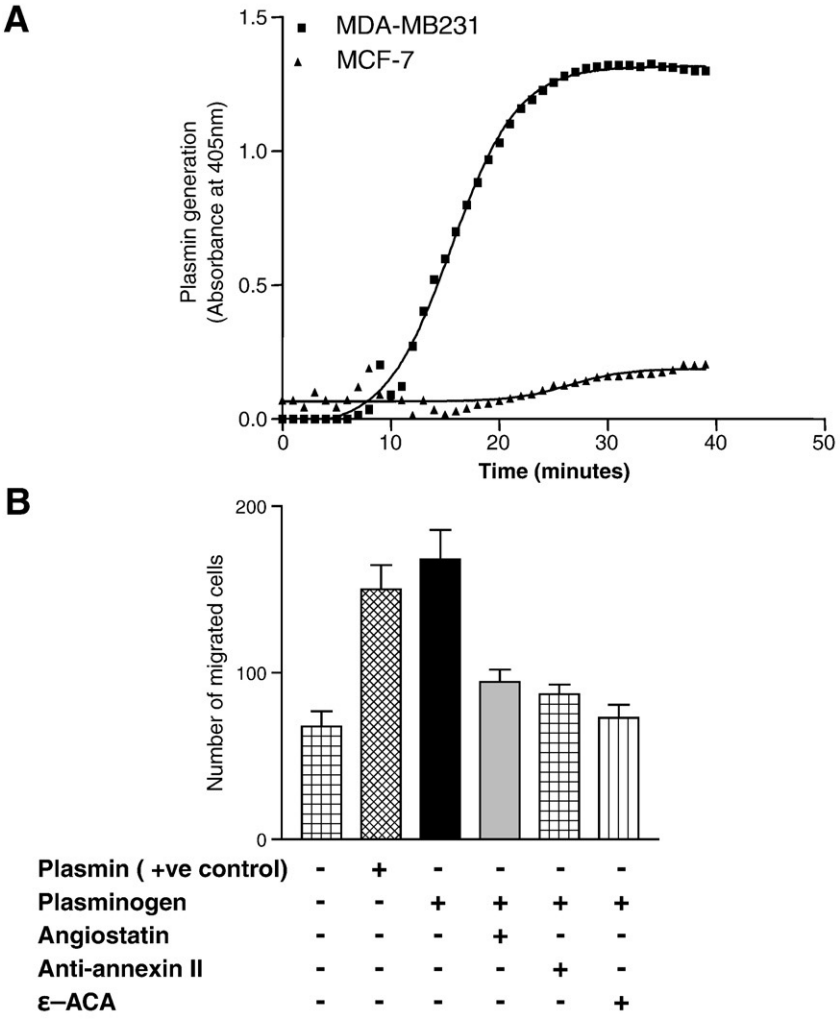

Fig. 2. Interaction of tPA with cell surface annexin II accelerate plasmin generation which in turn facilitates cell migration. MDA-MB231 cells were cultured in 12 well-plates. Cells were detached, washed and incubated and counted. About 20,000 cells were incubated with recombinant human tPA and allowed to bind on the cell surface annexin II for 30 min on ice. Cells were thoroughly washed to remove unbound tPA. tPA bound cells were incubated with plasminogen and chromozyme PL. Kinetics of plasminogen activation were monitored at 405 nm. Linearity of plasminogen conversion to plasmin was observed in about 20 min. Non invasive human breast cancer cells MCF-7 lacking annexin II failed to bind tPA and were unable to convert plasminogen to plasmin (A). tPA dependent plasmin generation induced cell migration. This enhanced cell migration could be blocked either by direct blocking of annexin II using anti-annexin II monoclonal antibodies or angiostatin which binds to cell surface annexin II and blocks plasminogen binding. Migration was also inhibited by blocking of lysine residue of plasminogen which is required for interaction with annexin II (B).

been correlated well with poor clinical outcome with advanced stages of cancers (Brichory et al., 2001; Roseman et al., 1994; Sharma et al., 2006a; Sharma and Sharma, 2007; Singh, 2007; Syed et al., 2007; Wu et al., 2002; Zimmermann et al., 2004). Because tPA is a ligand for annexin II and known to activate cell surface bound plasminogen to plasmin, we first tested whether annexin II and tPA proteins are synthesized by human breast cancer cells. Immunoblot analysis indicated selective expression of annexin II and tPA in highly invasive MDA-MB231cells but not in non invasive MCF-7 cells (Fig. 1A and B). Next we investigated whether tPA synthesized by MDA-MB231 cells interacts with annexin II. To determine interaction, we immunoprecipitated an MDA-MB231 cell lysate with anti-tPA monoclonal antibody, and the immune complex was resolved on SDS-PAGE and transferred onto nitrocellulose membranes. Bound proteins were identified by anti-annexin II monoclonal antibody by immunoblotting. Immunoblot analysis identified tPA bound protein as annexin II indicating that tPA synthesized by MDA-MB231 cells binds to annexin II (Fig. 1C). These results were confirmed by a converse experiment where lysate was immunoprecipitated by anti-annexin II monoclonal antibody and bound protein was recognized by anti-tPA antibody confirming that tPA binds to annexin II (Fig. 1D).

tPA binding to cell surface annexin II is required for plasminogen activation in breast cancer

The assembly of tPA and plasminogen on the cell surface annexin II facilitates conversion of inactive plasminogen to reactive enzyme plasmin. To test whether interaction of tPA and annexin II in MDA-MB231 cells are physiologically active, we assayed plasminogen activation in tPA bound live cells. MDA-MB231 cells were incubated with recombinant tPA and analyzed for cell surface binding. Unbound tPA was removed by washing. In parallel experiments non-invasive MCF-7 cells that lack annexin II expression were used as control. Specific tPA binding was determined by its ability to convert plasminogen to plasmin. Indeed, tPA bound to the MDA-MB231 cell surface and was able to activate plasminogen into plasmin in a time-dependent manner. As expected, MCF-7 cells lacking annexin II expression therefore tPA were unable to bind and consequently failed to activate plasminogen (Fig. 2A). To further confirm if cell surface interaction of annexin II and tPA is critical to activate plasminogen, we silenced annexin II gene in MDA-MB231 cells (Fig. 3A).These annexin II silenced cells were analyzed for their ability to bind tPA and their capacity to activate plasminogen. Results shown in Fig. 3C indicate that annexin II silenced MDA-MB231 cells were unable to bind tPA. Therefore activation of plasminogen was significantly inhibited. In contrast, wild type cells bound with recombinant tPA that activated plasminogen to plasmin efficiently (Fig. 3C). Collectively, these results strongly suggest that MDA-MB231 cell surface annexin II is required for tPA binding and is required for efficient plasmin generation.

Annexin II mediated plasmin generation is crucial for cell migration

Cell motility is critically involved in metastasis, neoangiogenesis and tumor invasion (Stack et al., 1999). It has been reported that plasmin is a strong serine protease and known to hydrolyze ECM and basement membrane (BM) proteins and thereby facilitates cellular migration, invasion and angiogenesis (Bajou et al., 2001; Diaz et al., 2002; Pepper, 2001; Tarui et al., 2002). Several studies were performed to determine the ability of annexin II to influence breast cancer cell migration. First we used a Boyden chamber assay model and tested if annexin II dependent plasmin generation is required for MDA-MB231 cell migration. The results of this experiment revealed that when cells were incubated with plasminogen they activated plasminogen to plasmin which in turn degraded ECM proteins and invaded across the membrane (Fig. 2B). This cell migration was significantly blocked by anti-annexin II antibody (Fig. 2B) suggesting that annexin II is involved in plasmin generation and its dependent cell migration. Additional experiments were performed to confirm that annexin II-dependent plasmin generation plays a critical role in breast cancer cell migration. Previously we have shown that

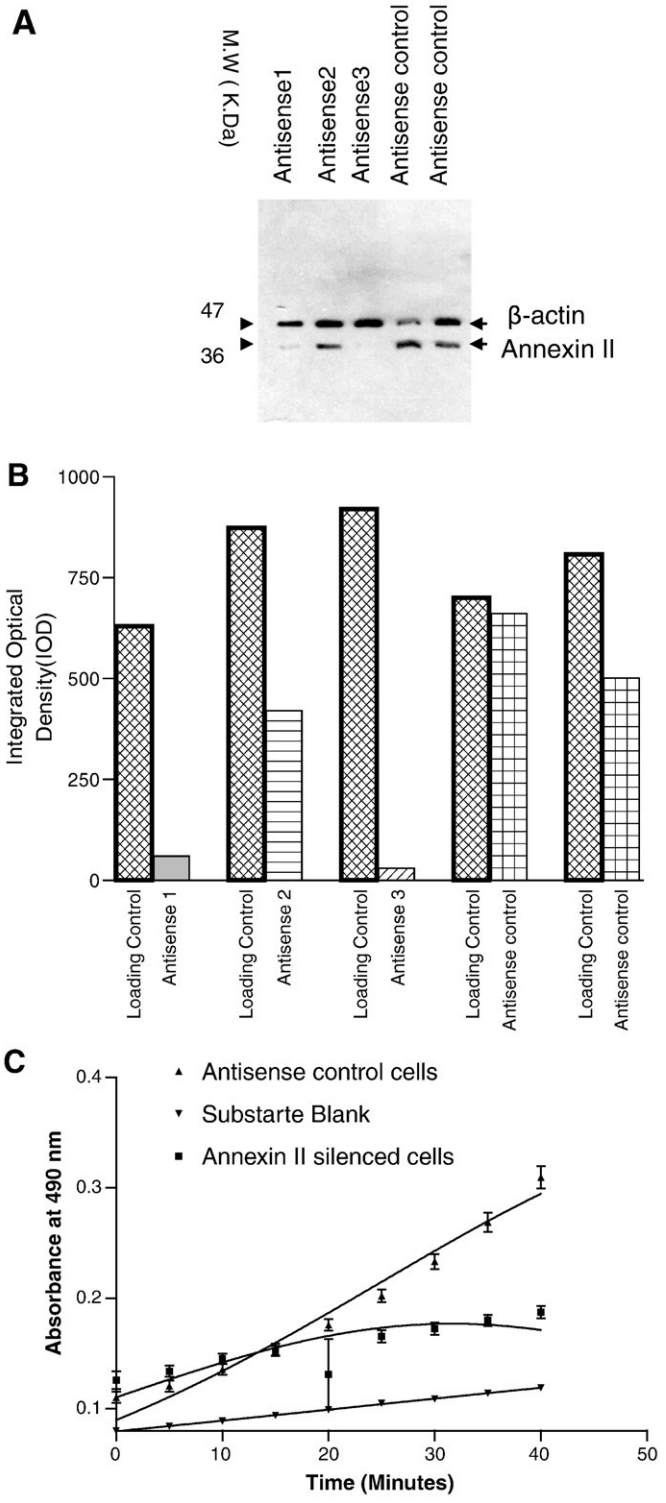

Fig. 3. Annexin II gene silencing inhibits tPA dependent plasmin generation. Three different antisense oligos were designed and synthesized as 16-mer dithionated oligonucleotide to silence annexin II gene. Immunoblot analysis suggests that two out of three antisense oligos were highly potent and almost completely silenced annexin II expression (A). β-actin was used as a loading control to confirm equal protein loading. Quantitative analysis of band densities showed more than 90% inhibition of annexin II expression as compared to β-actin loading control (B). Annexin II-silenced cells failed to bind tPA and significantly inhibited plasminogen activation (C).

angiostatin competes with plasminogen binding on endothelial cell surface annexin II because angiostatin and plasmimogen share common binding sites and angiostatin competes for plasminogen binding (Tuszynski et al., 2002). We speculate that angiostatin may inhibit cancer invasion and migration (Stack et al., 1999; Tarui et al., 2002) by inhibiting plasmin generation. To test whether angiostatin blocks cell migration, we incubated angiostatin with the MB231 cells prior to plasminogen and examined whether this peptide inhibits annexin II-mediated breast cancer cell migration. As shown in Fig. 2B angiostatin indeed inhibited MDA-MB231 cell migration significantly suggesting that angiostatin blocks annexin II-dependent plasmin accumulation which in turn inhibits cell migration. Since lysine binding domains in plasminogen is critical for binding to cell surface receptors and activation to plasmin (Felez et al., 1996; Tuszynski et al., 2002), we next investigated the role of these residues in breast cancer cell migration using a lysine analogue ε-aminocaproic acid (EACA). Interestingly addition of EACA inhibited breast cancer cell migration significantly. These results support the conclusion that effective blocking of annexin II dependent plasmin generation may be critical for MDA-MB231 cell migration. In a second approach we silenced annexin II gene in MDA-MB231 cells (Fig. 3A) and assayed their abilities to activate plasminogen as well as migratory potential in a scratch wound healing model of cell migration. Results presented in Fig. 3C shows that silencing of annexin II expression significantly inhibited tPA binding and its dependent plasminogen activation. Annexin II silenced cells showed significant lower migratory activity as compared to wild type cells confirming that annexin II dependent plasmin generation is indeed required for MDA-MB231 cell migration (Fig. 4A and B).

Discussion

Tumor progression and metastasis involves degradation of ECM and BM proteins. Once this anatomic barrier is destroyed tumor cells start invading into adjacent host tissue and normal host cells start migrating into the tumor. Such migratory, invasive and tissue remodeling events are regulated by different proteolytic enzymes (Pepper, 2001). Among the proteases that play an active role in these processes is the serine protease plasmin. Plasmin is an end enzymatic product of plasminogen/plasmin pathway. Physiological roles of plasmin were believed to be limited to fibrinolysis, extracellular proteolysis, migration of macrophages to a site of injury, tissue repair/remodeling, invasion and angiogenesis (Lijnen, 2001; Pepper, 2001). Many advanced human tumors including breast cancer overproduce plasmin that is known to promote neoangiogenesis, cancer invasion and metastasis (Paciucci et al., 1998; Sharma et al., 2006a). Analysis of the enzymes of plasminogen/plasmin system suggests that high urokinase-type plasminogen activator (Li et al., 1998) in breast tumors predicts poor survival and is associated with a high rate of relapse for patients with breast cancer (Janicke et al., 2001; Janicke et al., 1989; Look et al., 2002). These clinical observations suggest that the components of plasminogen/plasmin system can contribute to tumorigenesis in a variety of tissue types (Ossowski and Aguirre-Ghiso, 2000) including breast cancer. Consistent with this notion, inhibition of uPA activity seems to inhibit breast cancer in a mouse model (Rabbani and Gladu, 2002). Existing literature clearly indicates that under pathological conditions uncontrolled production of plasmin in the tumor microenvironment can accelerate the localized degradation of ECM and BM. Destruction of this anatomical structure may lead to release of matrix bound latent growth factors such as bFGF and VEGF which are known angiogenic mitogens (George et al., 2001; McColl et al., 2003). Earlier studies showed that proteolytic activity of plasmin is required to activate these growth factors which reprogram migrating endothelial cells for proliferation to establish new blood vessels (McColl et al., 2003).

Considering this fact our *in vitro* data may be of immense importance which shows that annexin II on the surface of breast cancer cells could be a molecular switch that specifically binds with tPA and thus regulates plasmin generation (Figs. 1 and 2A). Since noninvasive breast cancer cells MCF-7 that lacks annexin II expression failed to activate plasminogen may suggest specificity of invasive MDA-MB231 cells in plasmin generation (Figs. 1 and 2A). Because our data show interaction of tPA with annexin II in MDA-MB231 cells (Fig. 1.) that is capable to produce plasmin (Fig. 2A), we sought to determine if plasmin is involved in breast cancer cell migration. The data presented in Fig. 2B show that the cell migration was indeed plasmin dependent because ε-aminocaproic acid, Lys analogue, which is known to disrupt kringle–lysine interaction between plasminogen and their receptors, inhibits plasmin generation and migration in Boyden chamber model (Felez et al., 1996; Tuszynski et al., 2002).

Previously we have shown that angiostatin competes for plasminogen binding on EC surface annexin II and blocks plasmin generation (Sharma et al., 2006b; Tuszynski et al., 2002). Moreover angiostatin was reported to inhibit neoangiogenesis dependent breast cancer growth and metastasis (Griscelli et al., 1998; O'Reilly et al., 1996; Peyruchaud et al., 2003) in mouse model. Our data demonstrates that blocking of annexin II either by angiostatin or monoclonal antibody to annexin II effectively blocks MDA-MB231 cell migration indicating specificity of annexin II in cell migration (Fig. 2B). Based on our experimental evidence it can be argued that angiostatin mediated inhibition of cell migration may be due its ability to inhibit plasmin

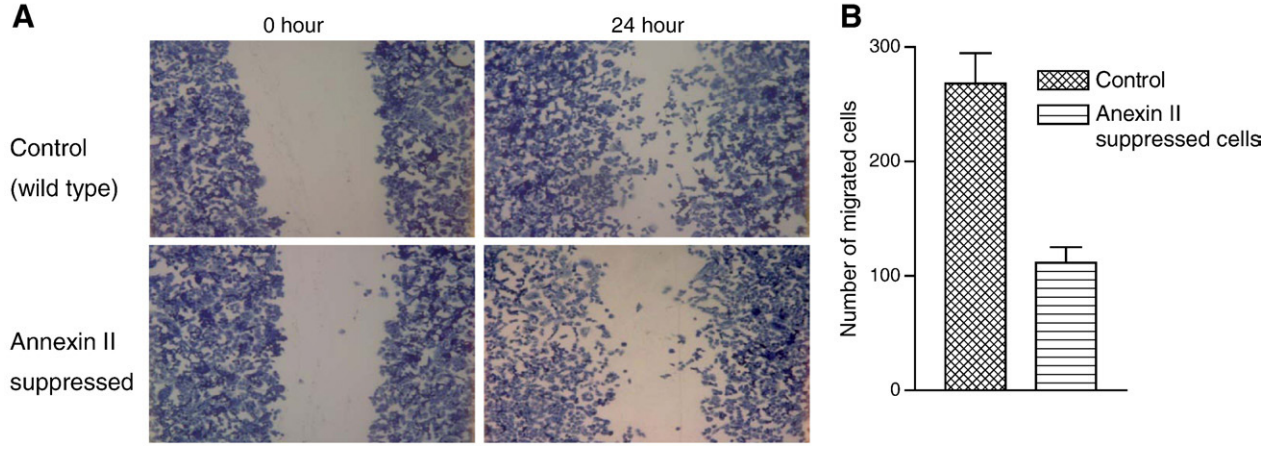

Fig. 4. Annexin II gene suppressed MDA-MB231 cells reduces cell motility. Annexin II gene silenced cells were grown in 96-well-plates. Cells were denuded by scraping and incubated for 24 h. After 24 h cells migrated across the wounded edge were counted and results are expressed as the mean ± SD of three experiments.

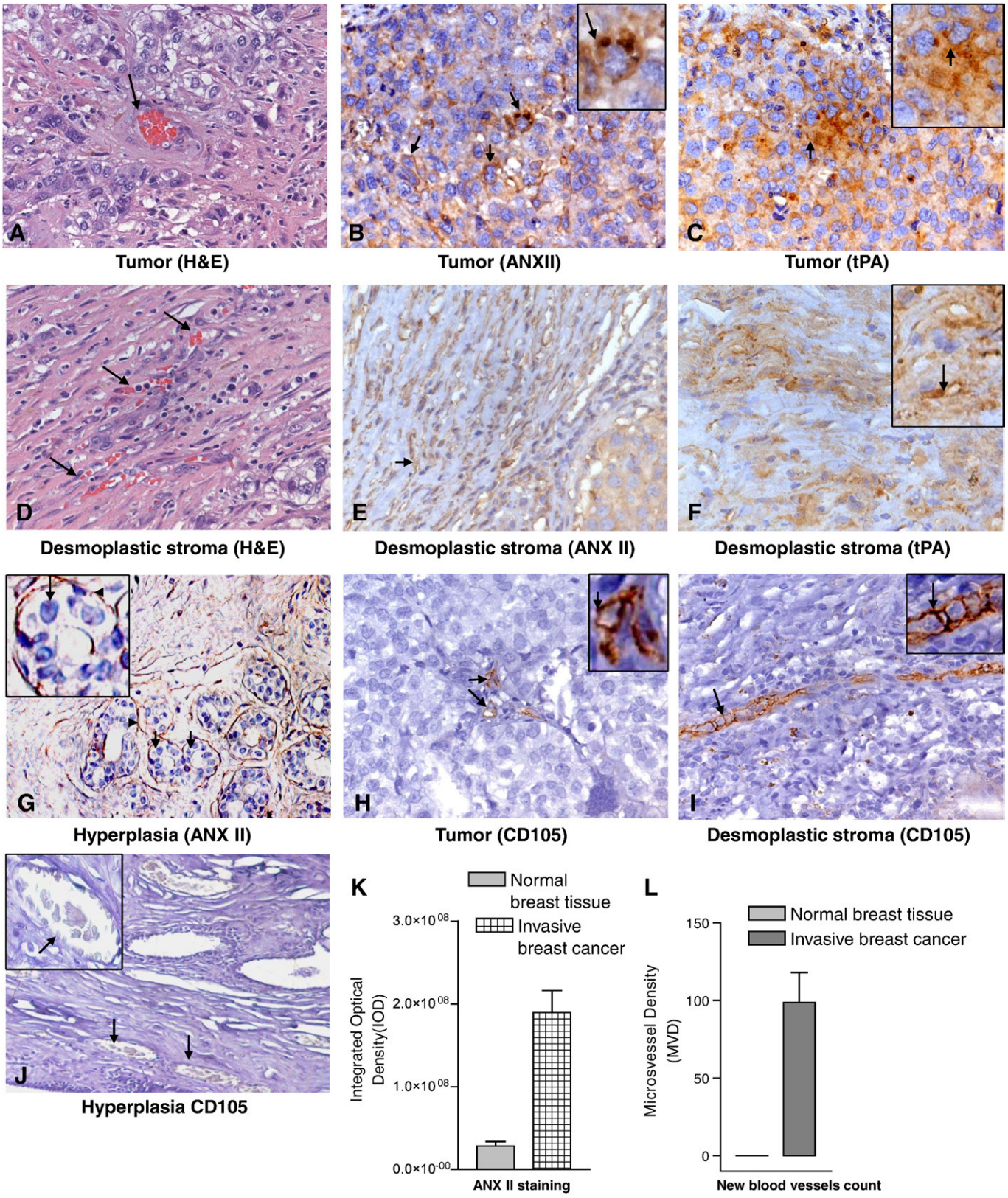

Fig. 5. Immunostaining for annexin II, tPA and anti-CD 105 in normal and human breast cancer tissues. Annexin II is undetectable in normal acinar and ductal epithelial cells (G, inset arrows). Anti-annexin II antibodies show a very strong reactivity on the surface of ductal epithelial cells (B, inset), tumor stroma (E) and vascular endothelial cells (B, arrows) in invasive breast cancer. Staining in ductal epithelial cells was found mainly on the cell surface with occasional cytoplasmic staining. Anti-tPA antibodies show intense staining in vascular endothelial cells(F inset), ductal epithelial cells (C, inset) and in tumor stroma. Anti-CD105 antibodies show a selective strong reactivity with newly formed blood vessels (H) but not in existing blood vessels of normal tissue (J). Panel J arrows indicate existing blood vessels in normal breast tissue are not stained with anti-CD105 antibody suggesting specificity of CD105 to identify proliferating endothelial cells (Magnification 20X). Staining intensity of annexin II was analyzed in 20 different fields by ImagePro Software Panel K. CD 105 positive endothelial cells were counted and plotted by GraphPad Prizm software Panel L.

generation by blocking plasminogen/tPA binding on cell surface annexin II (Brownstein et al., 2004; Stack et al., 1999; Tuszynski et al., 2002). Silencing of annexin II gene in MDA-MB231 cells inhibits their ability to bind tPA and plasminogen activation (Fig. 3). Furthermore these cells were less migratory as compared to wild type in a scratch wound healing model of migration assay (Fig. 4). Based on our data it is not surprising that Shiozawa et al. have found involvement of annexin II in adhesion, migration and homing of prostate cancer cells (Shiozawa et al., 2008). Together, these *in vitro* and *in vivo* experimental evidence links a potential role of annexin II in breast cancer.

It is possible that plasmin mediated destruction of the anatomic barrier of ECM and BM could support EC /cancer cell migration (Tarui et al., 2002) that may be the key for new blood vessel formation to support tumor expansion and metastasis (Ling et al., 2004; McColl et al., 2003; Semov et al., 2005). Our observations are further supported by the facts that tumors failed to grow on plasminogen (PLG-/-) gene knockout mice and unable to metastasize because in absence of plasmin tumor's ability to from new blood vessels was significantly inhibited (Bajou et al., 2001; Perides et al., 2006). Consistently a number of prior articles have described that direct inhibition of plasmin suppresses experimental metastasis, neoangiogenesis and tumor progression in Lewis Lung Carcinoma (LLC) model (Bugge et al., 1997; Tanaka et al., 1982).

Taken together, our data provides strong evidence that annexin II-dependent plasmin generation is required for breast cancer cell migration. It seems that annexin II is a regulatory switch for continuous plasmin generation in the tumor microenvironment and might explain the presence of annexin II on the cell surface of many different types of human cancers.

These data raised the possibility that annexin II dependent plasmin generation may be an endogenous regulator of neoangiogenesis (Ling et al., 2004). To investigate this we analyzed human breast tissue samples by immunohistochemical staining. Immunohistochemical studies revealed expression of ANX II mainly on the surface of invasive cancerous cells (Fig. 5B). In contrast, epithelial cells in normal ducts were completely negative (Fig. 5G). Consistent with annexin II expression, immunostaining of thin serial sections also revealed high levels of tPA in tumor stroma (Fig. 5C). In agreement with our findings earlier clinical studies have also noticed elevated levels of tPA in the plasma of human breast cancer (Grondahl-Hansen et al., 1990; Rella et al., 1993), glioblastoma and pancreatic cancers (Aguilar et al., 2004; Goh et al., 2005). Careful investigation of immunostained sections revealed collagenous stroma composed of fibroblasts, macrophages, lymphocytes and ECM indicating an inflammatory response and annexin II in desmoplastic stroma (Fig. 5E) suggesting chronic inflammation in the tumor microenvironment and may be indicative of neoangiogenic activity. Next, we investigated neoangiogenic activity in the serial sections of same tissue using anti-CD105, selective marker for sprouting neovessels (Gerber et al., 2006; Kumar et al., 1999). Staining pattern of CD105 revealed numerous sprouting neovessels in tumor as well in desmoplastic stroma confirming neoangiogenic activity (Fig. 5H and I). Quantitative analysis of neoangiogenic activity as measured by micro vascular density (MVD) appears to correlate with annexin II expression patterns (Fig. 5K and L).

Based on our *in vitro* and *in vivo* data, it is quite likely that over-expression of annexin II in human breast cancer regulates local plasmin generation which in turn degrades ECM and BM. Hydrolysis of matrix components by plasmin may release and activate matrix bound inactive angiogenic cytokine VEGF and may induce neoangiogenic activity (McColl et al., 2003). This could be an early event in the tumor microenvironment switching breast cancer from prevascular phase to the vascular phase (Ling et al., 2004) which is characterized by aggressive phase with the possibility of metastasis. Consistent with our observations neoangiogenic activity reported to be a significant prognostic indicator in early stage of breast cancer (Weidner et al., 1992).

Collectively, the data reported hear and previously published reports strongly support the concept that annexin II on tumor/EC may be an angiogenic switch. This may be a potential reason why annexin II is being repeatedly identified as widely overexpressed protein in number of cancers and strongly associated with prognostic significance in metastasis (Brichory et al., 2001; Chuthapisith et al., 2009; Guedj et al., 2009; Pei et al., 2007; Roseman et al., 1994; Sharma et al., 2006a; Sharma and Sharma, 2007; Shiozawa et al., 2008; Singh, 2007; Syed et al., 2007; Wu et al., 2002; Zhong et al., 2009; Zimmermann et al., 2004). Whether annexin II could be a valuable target in inhibiting neoangiogenic activity and breast cancer *in vivo* is in interesting but unanswered question that is currently under investigation in our laboratory.

Acknowledgments

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Inflammation, Microenvironment, and the Immune System in Cancer Progression

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Abstract: Since Virchow first proposed in 1863 that tumors could originate from sites of chronic inflammation, it has been well established that chronic inflammation both contributes to cancer progression and predisposes tissue to various types of cancer. Experimental, clinical, and epidemiological studies have all demonstrated the strong association between chronic inflammation and cancer, and many studies have correlated the prolonged presence of the inflammatory milieu with an increased risk for developing cancer. Proinflammatory cytokines, chemokines and adhesion molecules, which regulate the sequential recruitment of leukocytes, are frequently observed in tumor microenvironment. These early desmoplastic changes could stimulate fibroblasts and endothelial cell division and produce components for tissue remodeling and neovascularization, ultimately promoting neoplastic processes. In this review article we overview the current understanding of the role of chronic inflammation in neoangiogenesis, tumor initiation, promotion, and progression.

Key Words: Inflammation, microenvironment, neoangiogenesis, cancer.

INTRODUCTION

Neoplasia ("new growth") is the uncontrolled proliferation of transformed cells. The term *tumor,* which was originally used to describe the swelling caused by inflammation, is now used interchangeably with *neoplasm*. Transformation is the multi-step process in which normal cells acquire malignant characteristics. Each step in the neoplastic process reflects a genetic alteration that confers a growth advantage over normal cells. There are a number of essential alterations in cell physiology that collectively enable malignant growth: self-sufficiency in growth signals, evasion of programmed cell death (apoptosis), avoidance of immune detection and destruction, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. These characteristics are shared by most, if not all, human tumors. Despite tremendous progress in detection as well as treatment of cancer, rising cancer deaths requires a reevaluation of our basic understanding about the etiology of cancer and how to control it.

For the past four decades investigators have been trying to halt tumor growth by directly attacking cancer cells. This approach has provided limited clinical success coupled with a high degree of toxicity. Continuous failure has indicated that it is time to change our strategies and look beyond the cancer cells. Studies that have done such, through the implementation of tissue, gene and protein microanalysis, have shown significant alteration in the tumor microenvironment known as tumor stroma. These new investigations argue against our old understanding that carcinogenesis occurs in individual cells, and support a new idea that carcinogenesis occurs in tissue. The heterogeneous nature of the tumor is a result of continuous crosstalk between different cell types within tumor and its surrounding stroma.

Inflammation is the body's response to tissue insult. When tissue is damaged, either by physical trauma or by pathogen, a complex cascade of events is triggered. Numerous inflammatory cells and complexes collaborate to attack the invading pathogen, clear debris, reconstitute the extracellular matrix (ECM) and assist in the proliferation and transfer of healthy cells to the target site. The inflammatory response is often initiated by mast cells. When damaged, mast cells release histamine, an amino acid that increases the permeability of adjacent capillaries. Increased blood flow and vessel permeability enable the recruitment of inflammatory cells, including interleukins, neutrophils, cytokines and monocytes to the damaged tissue.

Ideally, inflammation is a self-limiting process, subsiding once the wounded tissue has healed. If inflammation does not subside within the normal course, the very mechanisms that the body uses to heal can promote unintended results, including neoplastic processes, such as cancer.

This heterogeneous nature of tumor stroma is very similar to granulation tissue formed during wound healing. Interestingly, Hal Dvorak in 1986 proposed that tumor build up their stroma by triggering host wound-healing response, which ultimately leads to fibrin deposition at the tumor site. This fibrin provides temporary ECM that forms a provision scaffold for cancer cells, inflammatory cells and newly formed blood vasculature. Thus he proposed the tumor to be "wounds that do not heal" [1].

Indeed, this relationship between chronic inflammation and cancer has been well established both *in vitro* and *in vivo*. For example, skin inflammation is associated with

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melanoma, bladder inflammation with bladder carcinoma, chronic pancreatitis with pancreatic carcinoma, chronic viral hepatitis with liver cancer, chronic HPV infection with cervical cancer, inflammatory bowel disease with colorectal carcinoma, smoking (which inflames the lungs) with lung cancer, and hepatitis C viral infection with hepatocellular carcinoma [2]. Components of inflammation have been reported to frequently be overexpressed and active within the tumor environment. The cancer-associated inflammation milieu is defined by the expression of: leukocyte infiltration, cytokines such as tumor necrosis factor α (TNF-α) and various interleukins, chemokines such as cyclooxygenase-2 (COX-2), monocytes derivatives called tumor-associated macrophages (TAM), matrix metalloproteinases (MMP), reactive oxygen and nitrogen species (ROS and RNS), and an activated nuclear factor κB (NF-κB) [3]. These components of cancer-associated inflammation work in a concerted effort to promote tumorigenesis in four major ways: by promotion of angiogenesis, by facilitation of cancer cell and tumor proliferation, by suppression of anti-tumor immunity, and by inhibition of apoptotic events.

In this article, we review the current literature supporting the relationship between chronic inflammation and cancer, as well as the current body of research that explains the possible mechanisms that define this relationship.

TUMOR ASSOCIATED MACROPHAGES (TAMs)

Derived from monocytic precursors circulating in the blood [4], tumor associated macrophages (TAM) are a significant component of inflammatory infiltrates in the tumor microenvironment [2]. In rare cases of breast cancer, TAM were found to represent more than 50% of tumor mass [5]. TAM concentration within the tumor environment appears to play a significant role in patient prognosis: a significant difference exists between the survival rates of patients with tumors defined as having low TAM density compared to patients with tumors defined as having high TAM density [6]. It was previously believed that optimally stimulated TAM were capable of killing *in vitro* tumor cells. This theory has been replaced with a general acceptance that TAM mostly, although not always, serve pro-tumoral functions [4]. For example, when activated by interleukin 12 (IL-12) TAM regulates T-cell dependent eradication of tumors [7]. However, it is more common for TAM to undertake the following pro-tumoral roles: assuaging tumor survival in hypoxic conditions due to angiogenesis, suppression of anti-tumor immunity, and aiding in neoplastic cell proliferation [8].

Development of a high density vessel network which connects the tumor to host circulation, termed the 'angiogeic switch' is crucial for the tumor's progression to malignancy [9]. Peri-tumoral accumulation of macrophages has been associated with blood vessel density in tumors [10]. Additionally, it has been demonstrated in mice that macrophages infiltrated the tumor site during the nonmalignant adenoma stage [10], the stage associated with the angiogenic switch. Together, these findings indicate that macrophages have a more significant role on tumor progression to malignancy rather than tumor growth. One suggested mechanism behind TAM infiltration and the subsequent triggering of angiogenesis states that tumors induce hypoxic stress, which in turn recruits TAM [7]. Once arriving at the site of hypoxia, TAM become angiogenically active; producing multiple proangiogenic growth factors and proteinases such as vascular endothelial growth factor (VEGF), matrix metalloproteinase-9 (MMP-9) and uPA [9].

TAM have been shown to suppress immune response through both poor antigen-presenting activity [11] as well as inhibition T-cell proliferation. TAM inhibits T-cell proliferation through the release of prostaglandins, IL-10 and TGF-β [11]. Prostaglandins have been established to suppress T-cell proliferation in various malignant disorders including Hodgkin's disease [12]. T-cells that are recruited to the site of tissue injury also have diminished anti-tumoral capabilities as the result of TAM, as TAM have been shown to release chemokines that recruit T-cells that lack any cytotoxic function. As the cellular source of the chemokine CCL18, TAM was found to have significantly higher concentrations in the ascitic fluids from patients with ovarian carcinoma versus nonovarian carcinoma patients [13].

The proliferative effects of TAM were demonstrated *in vivo* in mice with mammary cancer. The macrophage colony stimulating factor (CSF-1) has been observed within human breast carcinomas, and correlated with poor patient prognosis [14]. Using a mouse model, tumor progression was compared between mice containing an inactivating mutation in the CSF-1 gene and wild-type mice. Expression of CSF-1 in the wild-type mice accelerated tumor progression and increased pulmonary metastasis [7]. Based upon these findings, CSF-1 is implicated as promoting metastatic potential by regulating TAM infiltration at the tumor site. TAM has also been shown to promote neoplastic proliferation by producing and secreting neoplastic factors including IL-1, IL-10 and platelet-derived growth factor in to the tumor stroma [15]. Finally, TAM also produce MMP, which degrade all structural components of the extracellular matrix (ECM) [16], allowing for easier tumor infiltration and cell proliferation.

MATRIX METALLOPROTEINASES (MMPs)

Expression and activation of MMP, a proteolytic enzyme, is increased in almost all human cancers compared to noncancer tissue [17]. Furthermore, expression of MT 1-MMP and MMP2 are associated with poor prognosis in patients with various carcinomas [18]. In animal studies, up-regulation of MMP expression increases the aggressiveness of benign cancer cells, while down-regulation has the opposite effect. MMP appear to play a paradoxical role in cancer progression while acting in both pro and anti angiogenic roles. As a tumor promoter, MMP aids in neoplastic cell proliferation and immunosuppression.

MMP-1, MMP-2, MMP-9 and MT-MMPs are all known to promote angiogenesis through various mechanisms [19]. These mechanisms include control of endothelial cell tube morphogneis in three dimension ECM assay, regulation of vessel stabilization by controlling pericyte recruitment [19], and mediating fibrinolytic and collagenolytic activity [20]. Recent studies provide evidence that the pro-angiogenic effect of MT-MMP is mediated by an up-regulation of VEGF. VEGF expression, in turn, has been reported to be controlled

by MMP-9. MMP-9 controls VEGF bioavailability by cleaving it from the ECM [21]. Once released from the ECM, MMP-1, MMP-3, MMP-7 and MMP-13 selectively degrade connective tissue growth factor bound to VEGF, thereby increasing the reactive angiogenic activity of VEGF [21].

Conversely, MMP-7, MMP-12, MMP-13 and MMP-20 are believed to be negative regulators of tumor angiogenesis as a result of their capacity to generate angiogenic inhibitors. These angiogenic inhibitors are produced through the cleavage of plasminogen type IV or type XVIII collagens [22].

Similar to TAM, MMP have adverse effects on T-cell efficacy as an anti-tumor agent. Interleukin-2 receptor α (IL-2Rα) has a direct role in the production of functional T-cells. It has been demonstrated in cervical cancer, where tissues over express MMP-9, that MMP-9 mediates cleavage of IL-2Rα and reduces the functionality of cancer-encountered T-cells, ultimately decreasing the function of tumor-reactive cytotoxic lymphocytes [23]. This finding was supported by the restoration of T-cell proliferation when MMP inhibitors blocked MMP induced IL-2Rα cleavage.

MMP also affects cell invasion. E-cadherin is a cell-cell adhesion molecule responsible for epithelial cell aggregation along with the ability to suppress tumor invasion. Disturbance of E-cadherin resulting in a loss of cell-cell adhesion during tumor progression is positively correlated with tumor invasiveness [24]. An *in vitro* study conducted with a human breast cell line demonstrated that the MMP matrilysin and stromelysin 1 both cleave E-cadherin at the cell surface. It was concluded that this direct cleavage is capable of inhibiting epithelial cell aggregation, as well as facilitating invasion of epithelial cells in a paracrine manner [25]. MMP also act as a broad spectrum protease, indiscriminately degrading ECM and thus priming tissue for pathogen invasiveness [25].

MMP's role as a tumor promoter has been demonstrated both in humans and in mice. *In vivo,* mice lacking MMP-9 displayed reduced proliferation at all neoplastic stages and a decreased incidence of invasive tumors [20]. MMP-9 from bone marrow-derived inflammatory cells has also been proven to contribute to the HPV16 squamous carcinogenesis pathway [20].

TUMOR NECROSIS FACTOR A (TNF-α)

Once the inflammatory response has been initiated, tumors actively recruit inflammation-associated infiltrate cells, which upon arrival produce their own set of proteins: cytokines and chemokines [26]. One of the most prominent cytokines in the inflammatory infiltrate is TNF-α, a pro inflammatory cytokine that regulates growth development, differentiation, wound healing and immune response. TNF-α plays a dual role in tumor progression, switching between pro and anti-tumoral roles in a dose dependent manner. At high concentrations, TNF-α destroys tumor vasculature and induces tumor necrosis. When chronically produced in relatively low concentrations, as it frequently is in chronic inflammation, TNF-α acts as a tumor promoter [26]

TNF-α has been shown to play an especially important role in early stage tumor promotion both *in vitro* and in mouse models. TNF-α deficient mice developed 5-10% the number of tumors developed by wild-type mice during the initiation and promotion phase of tumor progression [27]. After the initiation and promotion phase, TNF-α deficient mice developed 25% the number of tumors as wild-type mice, indicating that TNF-α is integral in the initiation of the tumor as opposed to tumor growth [27].

TNF-α also affects tumor stroma through the induction of chemokines and MMP, and by stimulating fibroblast growth and function; all being angiogenic factors. TNF-α also expresses receptors for angiogenic cytokines. Working in concert with IL-1, TNF-α produces the angiogenic factor VEGF, while simultaneously destroying ECM. ECM is also degraded when TNF-α is chronically expressed as a result of TNF-α stimulated production of u-PA. Activated u-PA induces proteolytic cleavage of ECM, causing the release of additional pro angiogenic factors.

As mentioned herein, high concentrations of TNF-α can induce tumor necrosis. To combat this, tumors are capable of producing soluble TNF-α receptors. Produced by TAM, these receptors function as a defense mechanism, protecting the tumor from the tumoricidal and immunoactivating effects of TNF-α.

TNF-α also induces production of DNA damaging reactive oxygen species, while simultaneously inhibiting the ability of repair enzymes to repair damaged DNA. Further damage occurs to DNA when TNF-α is initially recruited to the damaged tissue site. TNF-α recruitment increases uptake of oxygen at the damaged tissue site, which leads to the release of free radicals that interfere with healthy epithelial and stromal cells. Ultimately, the processes that result from an environment characterized by persistently low TNF-α concentration lead to carcinogenesis by disrupting targets and pathways that the body requires to maintain homeostasis.

INTERLEUKIN FAMILY

Another pro-inflammatory cytokine that contributes to cancer is interleukin 6 (IL-6). Along with IL-4 and IL-10, IL-6 is highly expressed in hormone refractory prostate cancer. Additionally, prostatic carcinoma usually metastasizes IL-6 rich tissues sites, such as the liver, lymph nodes and bone [28]. Hodgkin lymphoma shows high levels of IL-6, evidence of which is associated with poor patient outcome. It has been hypothesized that a heritable abnormality in IL-6 regulation may be a predisposition for young adult Hodgkin lymphoma [29]. *In vitro,* IL-6 has been shown to stimulate colony formation of Is reco 2, a cell line derived from liver metastasis, indicating that IL-6 may be a stimulator of metastatic colon carcinoma cell growth [30]. Inhibition of IL-6 production and subsequent IL-6 signal transduction as a result of TGF-β production in tumor infiltrating lymphocytes suppresses tumor growth in the colon [31]. It was demonstrated that IL-6 signal transduction was regulated by soluble IL-6R, and therefore, tumor growth is controlled by IL-6-trans-signaling *via* the soluble form of IL-6Rα. IL-6Rα is one of the two subunits that constitute the IL-6 heterodimer. The other subunit, glycoprotein 130 (gp130), triggers phosphorylation of STAT 3 protein, which has a well documented role in malignant cell proliferation and survival rates [31].

IL-6 is also indirectly induced by IL-1α and IL-1β, which are secreted by macrophages alongside TNF-α, and help directly initiate inflammation. IL-1α and IL-1β are also responsible for inducing other pro-inflammatory factors such as inducible nitric oxide synthase (iNOS) and cyclooxygenase type 2 (COX-2). Similar to TNF-α, IL-1 can both assist and inhibit tumor progression based on concentration within the tumor environment. When expressed in small quantities, IL-1 produces a tamed inflammatory response that can be suppressed by normal anti-tumor immunity. However, as a result of its paracrine effects on stromal cells in the tumor's microenvironment, large amounts of IL-1 can induce major metastasis that is not susceptible to anti-tumor immunity [32]. Indeed, high IL-1β concentration is correlated with other accepted parameters of aggressive tumors in advanced breast carcinomas [33].

CYCLOOXYGENASE 2 (COX 2)

As mentioned above, COX-2 is induced by pro-inflammatory cytokines. It has been demonstrated to promote tumor progression, and is up-regulated in cancers of the pancreas, [34] prostate, [35] colon, [36] breast, [37] lung, [38] as well as in multiple squamous cell carcinoma of the head, [39] neck, [39] and esophagus [40]. COX-2 aids tumor progression by induction of angiogenesis, facilitation of cell proliferation, and prevention of apoptosis

Two cytokines which induce COX-2 expression are the pro-angiogenic factors TNF-α and IL-1β. Using a mouse cornea model, it was demonstrated that COX-2 expression regulates IL-1β induced angiogenesis, as the corneas of COX-2 null mice induced less angiogenesis than the corneas of wild-type [41]. The mechanism behind this can be attributed to the biological function of COX-2. COX-2 is one of two isoforms of cyclooxygenase, an enzyme that catalyzes prostaglandin from arachidonic acid (AA). It is this COX-2 production of prostaglandins, specifically E2 (PGE2) and thromboxane A2 (TXA2) that contributes to promotion of angiogenesis [42]. Metabolism of AA in order to form prostaglandins has been shown to be significantly higher in malignant prostatic tissue versus benign tissue [43]. In the above mouse experiment, PGE2 receptor (EP2 and EP4) agonists and a TXA2 receptor agonist induced angiogenesis both *in vitro* and also in mouse cornea. IL-1B-induced angiogenesis is inhibited by a PGE2 receptor, EP4, antagonist and a TXA2 antagonist. Together these findings implicate COX-2 activated prostanoids as inflammatory angiogenesis regulators [42]. It is believed that both nonspecific and specific COX-2 inhibitors also provide an effective tool for reduction of angiogenic processes by inactivating MAPK and by blocking COX-2 production of VEGF [44].

During chronic inflammation, excessive COX-2 production of prostaglandins E2, PGE2 and PGI2 not only effects angiogenesis, but also enhances cell proliferation and invasiveness in both tumor and normal cells. When treated with COX-2 inhibitors, human colonic adenocarcinoma cell line (HCA-7) growth was reduced in colorectal carcinoma. It is interesting to note that COX-2 inhibition more effectively reduced the cancer growth when combined with HER-2/*neu* inhibitors [45]. COX has also been shown to disturb the balance between MMPs and tissue inhibitors of metalloproteinases (TIMP) [46]. A disruption of this balance can lead to over expression of MMP, which as discussed earlier has multiple pro-tumoral roles. COX role on cell invasiveness was also clearly demonstrated in prostate cancer progression. The hypothesis that COX metabolites including AA and PGE2 affect prostate cancer progression by increasing invasion of basement-membrane (BM) was supported by the subsequent inhibition of PGE2. Inhibition of this isoform reduced *in vitro* invasiveness by 85%, an effect attributed to PGE2 mediation of BM degradation [46].

It was also revealed that COX-2 specific as well as nonspecific nonsteroidal anti-inflammatory drugs (NSAIDS) caused apoptosis in a variety of cancer cells. A study using colon cancer cell lines showed that NSAID induced apoptosis was more severe in colon cancers expressing COX-2 [47]. Likewise, rat intestinal epithelial cells (RIE) expressed elevated COX-2 protein levels and demonstrated increased resistance to induced apoptosis. Effects were reversed by administering sulindac sulfide, a COX inhibitor [18]. These findings demonstrate the significance of the COX-2 pathway in apoptosis regulation in many cell types, and the potential of NSAIDS to reverse the effects of this pathway [44]. The preventative role of COX-2 in apoptosis is attributed to two possible mechanisms: apoptosis could be mediated by generation of prostaglandin products or by the removal of the substrate AA [44]. By inhibiting COX-2, AA metabolism is mitigated in order to stimulate the production of ceramide, a mediator of apoptosis [3].

INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS) AND REACTIVE OXYGEN SPECIES (ROS)

COX-2, a stimulator of proangiogenic factors and vascular permeability, is activated by nitric oxide (NO). NO is a major component produced by iNOS, and is induced by a combination of cytokines including TNF-α and IL-1β [48]. Increased expression of iNOS has been observed in multiple inflammatory conditions associated with cancer, such as Crohn's disease, H-Pylori gastritis, ulcerative colitis and viral hepatits [49]. NOS activity correlates with lymph node metastases, indicating that increased metastatic behavior is associated with high NOS activity [50]. iNOS production of NO has been documented to directly damage DNA by causing DNA lesions [51]. NO becomes more reactive with DNA when exposed to reactive oxygen species (ROS), transforming into a reactive nitrogen species (RNS). RNS can also inactivate enzymes that are critical to DNA repair. In high concentrations, as is often the case with chronic inflammation, RNS inhibits p53 function, a protein that induces cell death in damaged DNA [49]. The combination of these events creates an accumulation of damaged DNA, enhancing the invasiveness of cancer.

NO are also centrally involved in angiogenesis. It has been demonstrated that tumor produced VEGF requires a NO pathway within the endothelial compartment to promote neovascular growth [49, 51]. Specifically, NO activates COX-2, which as discussed can stimulate the production of proangigoenic factors and prostaglandins resulting in increased vascular permeability [52]. Additionally, NO medi-

ates an giogenesis induced b y p latelet activating f actor (PAF), a s w ell as angiogenesis i nduced by TNF, a fa ctor mediated by produc tion of P AF [53]. Inhibitors of NO s ynthase have also been shown to suppress angiogenesis [54].

NUCLEAR FACTOR κB (NF-κB)

Chronic i nflammation i s a ssociated wit h pe rsistent a ctivation of ge ne-transcription f actor NF -κB, whic h ha s be en implicated i n cancer progre ssion [55]. S pecifically, NF -κB activation ha s be en a ssociated w ith s everal aspects of t umorigenesis including prom oting c ancer c ell proli feration, preventing apoptosis, i nducing R NS, and i ncreasing tumor angiogenic and m etastatic pot ential [44]. NF -κB is b elieved to prom ote cancer duri ng t he tumor's prom otion pha se as opposed to the tumor initiation [56]. During the tumor initiation phase, degrees of proliferation, hyerploidy and dysplasia were found t o be c omparable i n m ice w ith a ctive NF -κB when compared to mice with inactivated NF-κB. As the mice matured, a discernable difference was noticed between mice with active NF-κB versus inactive NF-κB. Thus, inactivation of NF -κB ac tivity in the h epatocytes led to a d ramatic d ecrease in tumor promotion [56].

During t umor prom otion, a colitis-associated c ancer model d isplayed i ncreased epithelial cells a fter de letion o f IκB in in testinal ep ithelial ce lls. I kB k inase is th e p athway responsible for c ytokine activation of NF -κB. While inflammation r emained co nstant, there w as a dramatic decrease in tumor i ncidence [57]. F urther supporting t he a rgument that NF-κB a ctivation is i ntegral for m alignant conversion, as opposed to early stages or initiation, is the link that NF -κB assists in the survival of hepatocytes and their progression to malignancy [55]. Selective deletion of NF-κB in hepatocytes induced programmed c ell de ath of he patocytes t hat h ad undergone malignant transformation, and reduced the incidence of liver tumors [55].

The RelA (p65) s ubunit of NF -κB is required for i nduction of T NF-α d ependent ge nes [58]. Treatment of RelA-deficient m ouse fi broblasts a nd m acrophages wit h T NF-α resulted in s ignificant re duction in via bility, wh ereas RelA positive cells were unaffected. Cytotoxicity to both cell types was m ediated by T NFR1. R eintroduction of Re lA into the RelA null fibroblasts resulted in enhanced survival, demonstrating th at the p resence o f R elA is r equired for p rotection from TNF-α [58].

NF- κB's effect on a ngiogenesis ha s be en di splayed in human ovarian can cer ce lls, w here NF-κB bl ocking h as shown t o s uppress angiogenesis i n hum an ova rian c ancer. NF-κB signaling blockade also inhibited both *in vitro* and *in vivo* e xpression of VE GF and IL -8, t wo angiogenic f actors [59].

CONCLUSIONS

The ev idence r eviewed in th is ar ticle d emonstrates that chronic inflammation re sults in t he produc tion of m ultiple factors that can either stimulate or inhibit tumor growth and progression by m odulating tumor microenvironment. F or this re ason, a n effective t herapeutic s trategy m ay i nvolve normalizing t he func tion of t umor s troma i ncluding m acrophages, granulocytes, l ymphocytes, e ndothelial c ells and their associated pericytes, as well as fibroblasts in the microenvironment. Most proinflammatory c ytokines produc ed by either h ost i mmune c ells o r tumor c ells themselves, h ave been demonstrated to prom ote tumor de velopment. By contrast, proapoptotic (TRAIL) and anti-inflammatory cytokines (IL-10, TGF-β) interfere with tumor development itself. This illuminates t he c omplicated and p aradoxical role of inflammation in tumor d evelopment. E ach major p layer in the inflammatory proc ess ha s the a bility to bot h prom ote m alignant t umor progre ssion, as we ll a s aid in t he body's fi ght against cancer. As d escribed above, duri ng c hronic inflammation m any p athways are either activated o r d eactivated, leading t o m odulation of t umor microenvironment m ilieu. Because s ignaling p athways in th is m ilieu ar e either r egulated by hos t i mmune system or by t umor c ells, it has been difficult to identify a single component of the inflammatory pathways in t he m icroenvironment. Howe ver, re cent a ttempts to target tumor microenvironment have yielded exciting results. For example, statins reduce cancer incidence, and it has b een s uggested t hat pos sible m echanisms for t his e ffect involve the suppression of inflammation and angiogenesis in the microenvironment [60]. Studies have further shown that the chronic use of NS AIDs, which is already known to reduce colon c ancer, [61] i s a lso associated w ith a striking reduction in the risk of bre ast cancer [62]. This study found an a pproximately 50% re duction i n bre ast c ancer ri sk with aspirin us e, a nd re lative ri sk dropped t o 0. 29 wit h s pecific COX2 i nhibitors. T hese re sults une quivocally s upport t he notion t hat ta rgeting tumor m icroenvironment m ay be an effective s trategy f or th e tr eatment o f cancer. T his s trategy may prove more effective by targeting m ultiple m olecules using a c ombinatorial a pproach i n t he tumor m icroenvironment.

Pathogenic i nfection c auses acute i nflammation whic h initiates sequence o f ev ents such as ac tivation o f m ast cells, macrophages, and de ndritic c ells wh ich i n t urn fa cilitates entry of gra nulocytes. This event is followed by furt her recruitment of m acrophages. These s equential e vents set t he stage for i nfiltration of i mmune cells (lymphocytes) to initiate immune response against tissue injury. Finally, the tissue remodeling proc ess b egins a t t he i njury s ite by re cruiting mesenchymal c ells such as endothelial cells and fibroblasts. These c ells s ynthesize collagen an d ex tracellular m atrix (ECM) also form new b lood ve ssels (neoangiogenesis). Ultimately, t he pa thogen is destroyed, and the da maged ti ssue is repaired. Therefore, all sequences of events recede to their normal state and homeostasis maintained. Contrast to orderly and organized sequence of events, carcinogenesis is the chaotic di sorganization of inflammation and re pair m echanism as de scribed i n F ig. (**1**). S ince carcinogenic proc ess be gins with c ell m utation a nd hype r prol iferation, inflammation never e nds (c hronic i nflammation). Unre solved inflammation l eads t o chronic i nflammation and homeostasis i s not achieved. T his di sruption of t issue hom eostasis m echanism can in turn lead to another wave of inflammatory re sponse, which is trying to repair injured tissue by furt her promoting neoangiogenesis. T his c ontinuous ne oangiogenesis l eads to invasive a nd m etastatic s tate of c ancer. T his is c onsistent with t he idea propos ed by H al Dvora k that "tumors a re wounds that do not heal".

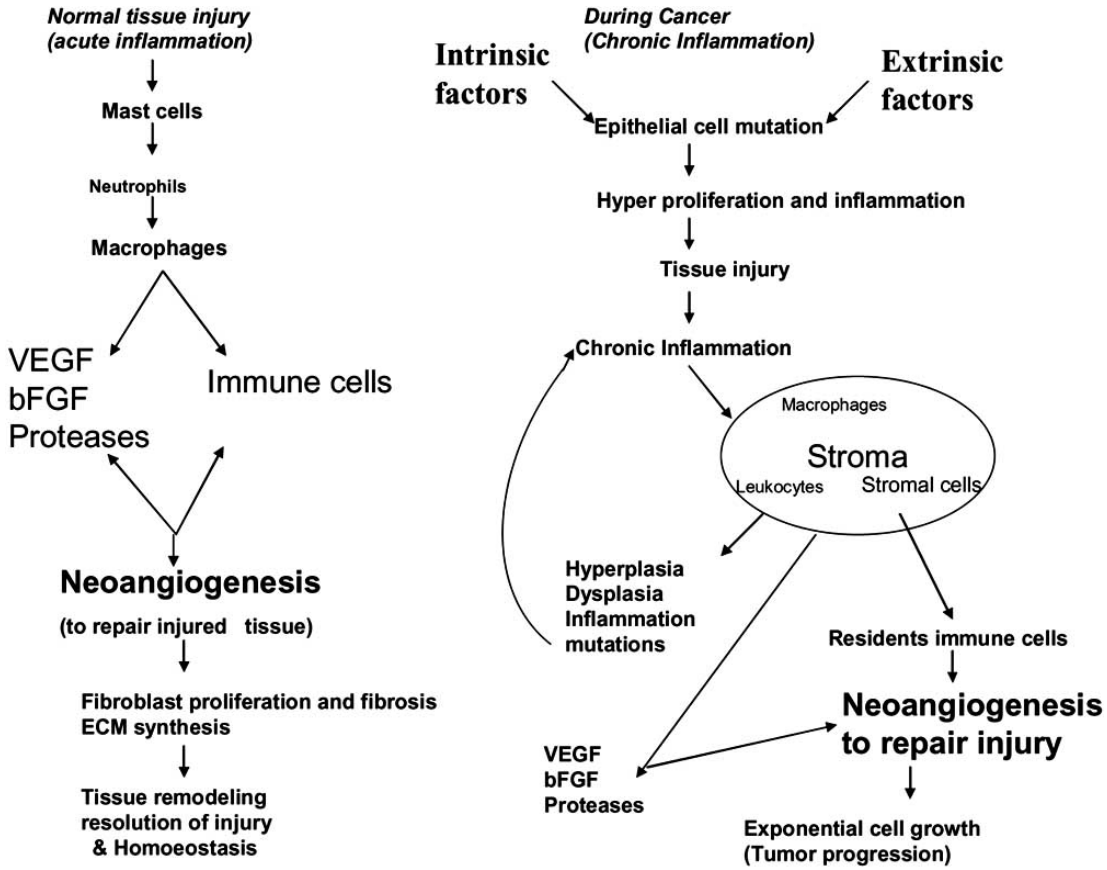

Fig. (1). Inflammation and tissue repair mechanism during normal tissue injury (acute inflammation) and cancer induced tissue injury (chronic inflammation).

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Inhibition of human breast cancer cell (MDA-MB231) migration, neoangiogensis and tumor growth by selective disruption of annexin II function: in vitro and in vivo studies

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Annexin II, an abund ant phosph olipid b inding cell surface protein, binds tissue plasminogen activator (tPA) and functions as a regulator of fibrinolys is. Annexin II also mediates angiogenesis and enhances tumor grow th and m etastasis. Ho wever, the m echanism supporting this role is not known. Using a hum an breast cancer m odel we found that invasive human breast cancer cells (MDA-MB231) synthesize annexin II and tPA . Annexin II interacted with tPA *in vitro* leading to conversion of zymogen plasminogen to the reactive enzyme plasmin. Cell surface generated plasm in inhibited the m igration of MDA-MB 231 cells. Silencing of the annexin II gene in m DA-MB231 cells abolished tPA binding thereby in hibiting tPA dependent plasmin generation. Moreover these annexin II suppressed MDA-MB231 cells exhibited reduced motility. Im munohistochemical analysis of pred iagnosed clinical specim ens showe d abundant secretion of tPA and expression of annexin II on the surface of invasive cancer cells which correlates with neovascularization of the tumor. Intravenous administration of the anti-annexin II antibody to m ice bearing MDA-M B231 tum ors signi ficantly inhibited tum or growth possibly due to blocked neoangiogenic ac tivity. Taken together, these data suggest that annexin II dependent plasm in generation may be an early event switching breast cancer from the prevascular phase to the vascular phase with the possibility of metastasis. Annexin II m ay be an attractive target for therapeutic strategies ai med to inhibit neoangiogenesis and breast cancer metastasis.